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A common structural motif in immunopotentiating peptides with sequences present in human autoantigens. Elicitation of a response mediated by monocytes and Th1 cells

Natalia López-Moratalla, Elena Ruíz, María J. López-Zabalza, Esteban Santiago *

Department of Biochemistry, University of Navarra, Pamplona, 31080, Spain

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Abstract

We have found a common structural motif in human autoantigens, heat shock proteins and viral proteins. Peptides modelled after sequences present in those molecules were synthesized and immunomodulating properties tested. They share a core of 15 amino acid residues and a common pattern ('2-6-11' motif) characterized by requirements at fixed positions with respect to a Pro (position 6); an apolar residue or a Lys at position 2; and a Glu, Asp or Lys at position 11. Any of these peptides, when added to cultures of lymphomononuclear cells, caused the activation of monocytes manifested by a release of IL-1α, IL-1β and TNFα. A release of INFγ and IL-2 took also place; this release was abolished by anti-DR antibodies. Neither IL-4 nor IL-5 could be detected. This suggests a presentation by APCs and the appearance of cells with a Th1 phenotype. Monocytes and Th1 cells freshly obtained from 12 patients of Graves' disease, 8 of Hashimoto's disease and 8 of primary biliary cirrhosis exhibited activation features similar to those found in cells from healthy subjects incubated in the presence of peptides with a '2-6-11' motif and representing fragments of autoantigens. Their immunopotentiating properties suggest their involvement in the initiation or progression of the autoimmune response mediated by activated monocytes and Th1 cells.

Keywords: Autoantigen; Cytokine; Monocyte; Th1; APC; Autoimmune disease

1. Introduction

The presence of autoantibodies against specific 'self' structures is characteristic of autoimmune diseases. However, their involvement in the pathogenesis of the disease usually depends on the localization of the presumptive target antigen (reviewed in [1]). Autoantigens processed and presented by professional or nonprofessional APCs could lead to the activation of T helpers favoring in this way the initiation, or the progression, of an autoimmune attack. It is not infrequent to find macrophages and lymphocytes infiltrating target tissues in organ specific autoimmune diseases. The molecular mimicry between microbial or viral peptides and self peptides presented by APCs would explain that an infection is often associated with the onset of an autoimmune attack (reviewed in [2]). A similar mimicry between heat shock proteins (hsps) and certain autoantigens has been linked to their possible involvement in some human autoimmune diseases (reviewed in [3]). In a recent review Elson et al. [4] propose that organ-specific autoimmune diseases result from the stimulation of immunologically ignorant T cells; they suggest that cytokines associated with T helper 1 cells alter the processing pattern of antigens and that the novel peptides so produced, and not previously exposed by thymic dendritic cells, are presented leading to an activation of autoreactive T cells. The recruitment of nonprofessional APCs, such as macrophages, that have the processing machinery able to generate new antigen cleavage patterns, is another mechanism to produce peptides capable of triggering an autoimmune response.

The aim of this study was to investigate the possible existence of a common structural pattern in autoantigens capable of conferring immunopotentiating properties to

Abbreviations: Graves' disease (GD); Hashimoto's disease (HD); Primary biliary cirrhosis (PBC); Healthy subject (HS); *S. aureus* protein A (SpA); Antigen presenting cell (APC); Heat shock protein (hsps); Phytohemagglutinin (PHA)

* Corresponding author. Fax: +34 48 425649.

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Table 1
'2-6-11' motif in human autoantigens

Autoantigens	Ref.	Disease	PI *	Sequence	Position
Mitochondrial M2	[3]	Primary biliary cirrhosis	P1	AKILVPEGTRDVPL	241–254
			P2	FADYRPTEVTDLKP	270–283P2
Glycogen phosphorylase(M4)	[2]	Primary biliary cirrhosis	P3	ALERWPVHLLETLL	384–397
			P4	KVHINPNSLFDIQV	555–568
17α–hydroxylase	[3]	Addison's disease	P5	WLKIFPNKTLEKLK	220–233
			P6	NGNAGPDQDSELLS	275–288
TSH receptor	[1]	Graves' disease	P7	SVTALPSKGLEHLK	237–250
Thyroglobulin	[3]	Thyroid disease	P8	QVDAQPLRPCELQR	24–37
Thyroid peroxidase	[24]	Hashimoto's thyroiditis	P9	CLCADPYELGDDGR	823–836
H^+, K^+ ATPase	[24]	Autoimmmune gastritis	P10	GVTLRPDVYGERGL	81–94
			P11	SGLVDPSFGFEEGK	163–176
			P12	LKDMDPSELVEALR	679–692
Glutamic Acid Decarboxylase	[3]	Insulin-dependent diabetes	P13	GVPDSPQRREKLHK	506–519
Insulin receptor	[2]	Insulin-dependent diabetes	P14	LLRWEPYWPPDFRD	476–489 (a)
			P15	SARTMPEAKADDIV	89–102 (b)
			P16	GYLDQPDNCPERVT	491–504 (b)
Islet cell Ag_{512}	[25]	Insulin-dependent diabetes	P17	YINASPIIEHDPRM	373–386
Carboxypeptidase	[26]	Insulin-dependent diabetes	P18	RIHIMPSLNPDGFE	109–122
			P19	AVPYSPAAGVDFEL	385–398
Asialoglycoprotein receptor H1	[2]	Chronic active hepatitis	P20	FKNWRPEQPDDWYG	234–247
α–Collagen II	[1]	Rheumatoid arthritis (RA)	P21	GANGEPGKAGEKGL	540–553
			P22	GLRGLPGKDGETGA	558–571
			P23	GARGAPGERGETGP	747–760
			P24	GIVGLPGQRGERGF	912–925
KU (p70/p80)	[3]	Scleroderma	P25	GSDIVPFSKVDEEQ	317–330
snRNP U1	[3]	Systemic lupus erythematosus (SLE)	P26	LGPDGPDGPEEKGR	335–348
Sm-D autoantigen	[1]	SLE	P27	LVDVEPKVKSKKRE	80–94
Acetyl choline receptor	[3]	Myasthenia gravis	P28	DLVGIPGKRSERFY	203–216
			P29	LVFLLPADSGEKIS	296–309
LHCG receptor and LH receptor	[24]	Premature ovarian failure	P30	KLQALPSYGLESIQ	232–245
Histidyl-tRNA synthetase	[1]	Myositis	P31	EVIDTPVFELKETL	91–104
			P32	VLLQTPAQEGEEPW	239–252
Isoleucyl–tRNA synthetase	[1]	Myositis	P33	ILERFPGAYLKGKK	275–288
			P34	VVHQAPYFGAEDYR	329–332
			P35	YLINSPVVRAENLR	626–629
			P36	KVLIDPVSVQDKDT	785–798
			P37	VIHQDPEALKDIKS	852–865
Myeloperoxidase	[27]	Glomerulonephritis	P38	SLWRRPFNVTDVLT	132–145
			P39	FVRWLPAEYEDGFS	183–196
			P40	LKSLNPRWDGERLY	362–375
			P41	PLVLGPTAMRKYLP	396–409
Serine protease	[1]	Wegener's granulomatosis	P42	IKEFIPFSGFKSGT	70–83
C_1–inhibitor	[1]	Angioedema	P43	SVEKVPMMNSKKYP	296–309
IL–1α	[28] [29]	Schnitzler's syndrome, RA	P44	CLAGGPPSITDFQI	243–256
Glutamic acid decarboxylase-2	[30]	Stiff-man syndrome	P45	ELADQPQNLEEILM	158–171
			P46	EIIGWPGGSGDGIF	202–215
Oligodendrocyte-myelin glycoprotein precursor	[31]	Multiple sclerosis	P47	NLSTLPSGLQENII	45–58
Myelin-associated glycoprotein precursor	[29]	Multiple sclerosis	P48	LSNVSPELGGKYYF	104–117
			P49	VSCMVPDNCPELRP	157–170
hsp60	[32]	Rheumatoid arthritis	P50	KKQSKPVTTPEEIA	155–168
			P51	VVTEIPKEEKDPGM	543–557
hsp70	[3]	SLE	P52	RKFGDPVVQSDMKH	76–89
			P53	AVITVPAYFNDSQR	142–155
hsp90	[2]	Ankylosing spondylitis and SLE	P54	EGLELPEDEEEKKK	539–552
64 kDa protein	[33]	Autoimmune ophthalmopathy	P55	KKALGPRRNSDLGK	83–96
			P56	VKKNEPLMEKEAKD	243–256
			P57	SIFDEPLERVKNND	289–301

Table 1 (continued)

Autoantigens	Ref.	Disease	PI *	Sequence	Position
Amphiphysin	[34]	Stiff-man syndrome	P58	SVVIEPASNHEEEG	542–555
				TIGAEPKETTEDAA	560–573
Laminin B2 chain	[35]	Good pasture'syndrome	P59	LGKTLPCGLTKTYT	303–316
			P60	PVSGAPAPWVEQCI	370–383
			P61	HLMDQPLSVDEEGL	1107–1120
N-cadherin	[36]	Pemphigus	P62	KLSLKPTKTEESVK	120–133
			P63	TKLSDPANWLKIDP	535–549
			P64	SAAPHPGDIGDFIN	824–838
E-cadherin	[36]	Pemphigus	P65	LLLRSPLGSQERSE	13–23
			P66	HVHGAPAPPEKRPR	35–49
			P67	ILSQDPELPDKNMF	300–314
			P68	PIFVPPEKRVEVSE	480–493
			P69	SIILKPKMALEVGD	645–659
			P70	AVVKEPLLPPEDDT	731–745
SS-B/La	[37]	SLE	P71	PSKPLPEVTDEYKN	93–106
Ro/SS-A	[37]	SLE, Sjogren's syndrome	P72	LAVAEPAVYFKEQF	14–27
			P73	TLIVRPDNTYEVKI	173–186
			P74	PVIQNPEYKGEWKP	264–277
FcRII and FcRIII IgG	[38]	SLE, Sjogren's and syndrome systemic sclerosis	P75	QAAAPPKAVLKLEP	1–14
RNA polymerase	[39]	Scleroderma, SLE	P76	FIAEVPIIAIDWVQ	28–51
			P77	RLGLIPLISDDIVD	67–80
			P78	QAPYDPNGKPERFY	216–229
Laminin B	[40]	SLE, RA, and polymyalgia rheumetica	P79	FKTTIPEEEEEEEE	546–559
Viral protein hepatitis B virus DNA polymerase			Pv1	PLGFFPDHQLDPAS	10–23(CI)
			Pv2	TVELLPYLPSDFFL	11–24 (E)
			Pv3	LVDKNPHNTAESRL	363–376 (E)
			Pv4	IVAPLPIHTAELLA	707–720 (E)

* **PI**, Peptide identification.

potential fragments. Among the properties considered we focused on the activation of monocytes and T helper cells with a Th1 phenotype.

We have already reported the immunomodulating properties of a series of peptides with sequences present in *S. aureus* protein A (SpA), and extracellular matrix proteins. These peptides share a core of fifteen amino acid residues with a common structural pattern ('2-6-11' motif) consisting basically in the presence of certain amino acid residues at fixed positions with respect to a Pro (position 6); an apolar residue or a Lys at position 2; and a Glu, Asp or Lys at position 11. Any of these peptides, when added to cultures of human lymphomononuclear cells, were capable of activating monocytes [5,6]. More recently, we have also found that peptides with this pattern are presented by HLA-II inducing the differentiation of $CD4^+$ T cells towards a Th1 functional phenotype [7]. We report now the existence of this structural '2-6-11' motif in autoantigens, viral and heat shock proteins permitting them to be presented by APCs, thus triggering an immune response mediated by T cells with a Th1 phenotype; this could explain, at least in part, the attack against 'self' which takes place in autoimmune diseases. This idea received a further support after the observation here reported that patients of several autoimmune diseases have monocytes and Th1 cells in a state of activation similar to that induced by fragments of autoantigens with the '2-6-11' motif on a population of lymphomononuclear cells obtained from healthy individuals.

2. Material and methods

2.1. Peptide synthesis

Peptides were synthesized by the solid phase method as described [8] with the Fmoc modification [9].

2.2. Cell source and preparations

Lymphomononuclear cells were obtained from heparinized blood of volunteers, healthy subjects or patients, by centrifugation over Ficoll-Hypaque as reported in the literature [10].

2.3. Cell culture conditions

Cultures contained 10^6 cells per ml and peptide concentration was 30 μg/ml in RPMI-1640 medium (Biochrom, Berlin, Germany) supplemented with 10% autologous serum, 2 mM L-glutamine, penicillin (100 U/ml) and

streptomycin (100 μg/ml) in humidified atmosphere with 5% CO_2 at 37°C.

2.4. Anti-HLA-DR antibody

Anti-HLA-DR antibody was obtained as described in the literature [11].

2.5. Cytokine determination

Cytokine determination was carried out in culture supernatants by the quantitative sandwich enzyme immunoassay technique with commercially available kits (Genzyme, Cambridge, MA). For the determination of the different cytokines lymphomononuclear cells were placed on 16 mm diameter wells (2×10^6 cells/ml in each well). Peptides were added to give a ratio of 30 $\mu g/10^6$ cells. After the incubation period plates were centrifuged and the determination of the different cytokines carried in duplicates using 100 μl of the supernatant of each well; the sensitivity of these assays was 50 pg/ml for each cytokine. Whenever IL-2 had to be determined, specific antibody against its receptor (anti-h-rIL-2 Ab BT563, Biotest Pharma, Germany) was added to the medium (2.5 $\mu g/10^6$ cells) at the beginning of a 7-day incubation period, in order to avoid that binding of IL-2 to its soluble receptor could mask its release. When the incubation was carried out in the presence of anti-HLA-DR antibody, this was added to the wells after the cells to give a final concentration of 2.5 μg/ml. After 5 minutes the peptide was added.

2.6. TNFα and IFNγ gene expression.

For the preparation of RNA 20×10^6 lymphomononuclear cells obtained from healthy individuals were incubated for different periods in the presence of active peptides. Non-adherent cells were collected and centrifuged; pelleted cells were immediately disrupted in 4 M guanidinium thiocyanate, 25 mM Na_3 citrate, 0.2% sarkosyl, 2 mM 2-mercaptoethanol solution. Total RNA from each sample was pelleted by ultracentrifugation over a cesium chloride gradient (5.7 CsCl, 100 mM EDTA) for 22 h. The pellet was precipitated with absolute ethanol and resuspended in water. For cDNA synthesis Eppendorfs containing 2 μg of RNA were incubated for 5 min at 90°C and chilled on ice for 3 min. Samples were then incubated for 1 h at 37°C after adding 10 U of RNAsin, 1 mM deoxynucleoside triphosphates, 100 U M-MLV reverse transcriptase (Gibco BRL, UK), 100 pmol of hexamers as primers and 8 μl of reverse transcriptase buffer; final volume was 20 μl. After the incubation, reaction was heat-inactivated at 90°C for 5 min, and cDNA frozen at −20°C until use. The polymerase chain reaction (PCR) was carried out in an automatic CNA thermal cycler. The reaction mixture contained 5 μl of the isolated cDNA, 30 pmol of sense and antisense primer, 1 mM deoxynucleotide triphosphates and 0.25 U Taq DNA polymerase (Promega, Madison WI, USA) in a final volume of 100 μl. The cycle program was set to denature at 95°C for 1 min, to anneal at 52°C for 30 s and to extend at 72°C for 30 s for a total of 35 cycles. Electrophoresis of the PCR products was performed on 1.5% agarose gels containing 1 μg/ml ethidium bromide. As molecular weight markers λ and φx-100 were used. Primers were prepared with an Applied Biosystem 391 synthesizer, using published gene sequences.

2.7. Surface marker CD14

Immunofluorescence determination of CD14 was performed with a Coulter Epics flow cytometer using the monoclonal antibody Leu-M3 obtained from Becton-Dickinson (Mountain View, CA) as a conjugate of fluorescein isothiocyanate (FITC) under conditions already specified (6)

2.8. Statistical analysis

Data were analyzed by the ANOVA and Fisher's PlsD tests; a p value less than 0.05 was considered to be significant.

3. Results

3.1. '2-6-11' motif in autoantigens

Upon examining the structures of autoantigens, heat shock proteins and hepatitis B virus DNA polymerase we have found that a '2-6-11' motif is present in their sequences at least once as shown in Table 1. Peptides containing fifteen amino acid residues (14 of them representing sequences corresponding to the '2-6-11' motif and an extra valine at the C end) were synthesized in order to investigate their potential immunomodulating properties.

3.2. Cytokine release by lymphomononuclear from healthy subjects

Cytokines released to the medium were determined after incubating human lymphomononuclear cells in the presence of synthetic peptides corresponding to fragments of autoantigens, hsps and viral DNA polymerase and containing the '2-6-11' motif. Two peptides were used as positive controls and known from previous work to have immunomodulating properties [5,6]: NVLGAPKKLNESQAV (Pa1) not present in natural proteins, and peptide QVLASPGSCLDEFRV (Pa2) from α1 collagen. As negative controls several peptides lacking that motif were used: ADAQQNKFNKDQQSV (Pc1) from SpA; a second one with a sequence present in thyroglobulin, SGLYNPIVFSASGAV (Pc2), in TSH receptor, EELKNPQEETLQAFV (Pc3), and in hsp 60 SKGANPVIRRGVMLV (Pc4). In all these peptides the valine residue at

the C-end was added for convenience of the synthesis and it is not necessarily present in the sequence of the reference protein. We have tested a number of peptides using cells obtained from 18 different healthy individuals.

Fig. 1 shows the release of TNFα, IL-1β and IL-1α induced by the different peptides when lymphomononuclear cells were incubated in their presence at the end of a 24 h period. All of the peptides having the '2-6-11' motif induced a considerable release of these cytokines, thus reflecting their ability to activate monocytes. It may be seen a that a serine residue at position 2, or that of an aspartic acid at position 11 resulted in peptides with relatively low activity when tested with cells belonging to healthy individuals.

Fig. 2 shows the release of IFNγ and IL-2 induced by the different peptides, in the absence or presence of anti-DR antibodies, on lymphomononuclear cells belonging to

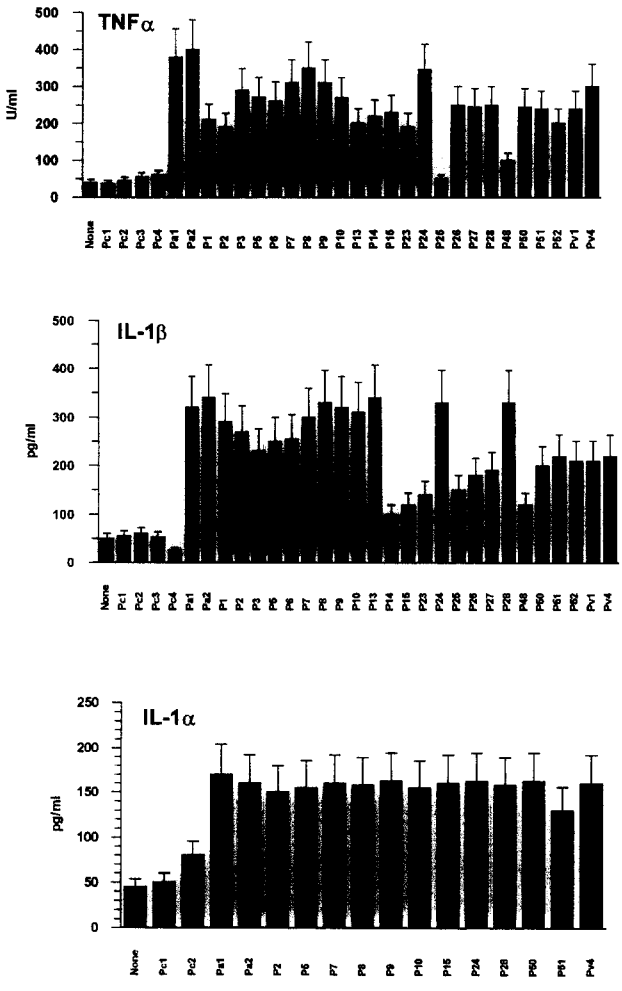

Fig. 1. Monocytic cytokines released by lymphomononuclear cells from healthy subjects after peptide activation. Results represent the mean of duplicates of 18 independent experiments using cells obtained from the blood of diferent healthy individuals. Differences between cells treated with immunomodulating peptides and cells treated with negative control peptides (Pc1, Pc2, Pc3, Pc4) or in the absence of peptides were statistically significant ($P < 0.001$). Peptide concentration was always 30 μg/ml. The incubation time was 24 h.

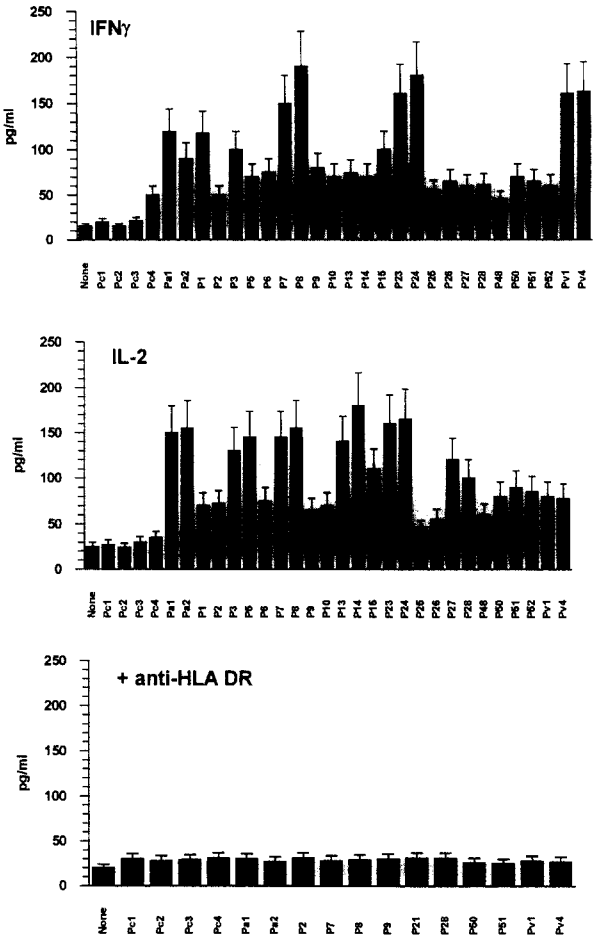

Fig. 2. Release of IFNγ (A) and IL-2 (B) by lymphomononuclear cells obtained from healthy subjects induced by immunomodulating peptides. Values represent the mean of duplicates of 15 independent experiments using cells obtained from the blood of different individuals. Differences between cells treated with immunomodulating peptides and cells treated with negative control peptides (Pc1, Pc2, Pc3, Pc4) or in the absence of peptides were statistically significant ($P < 0.001$). Peptide concentration was always 30 μg/ml. The incubation time was 24 h for IFNγ and 7 days for IL-2. (C) Effect of anti-HLA-DR antibody on IL-2 release induced by active peptides. The values represent the mean of 3 independent experiments. The concentration of antibody was 2.5 μg/ml.

healthy individuals. Both cytokines were released in amounts higher than those induced by negative control peptides or in the absence of any added peptides. The possible release of IL-4 and IL-5 to the medium was investigated, but its presence was never detected when lymphomononuclear cells obtained from healthy subjects were incubated for 24 h under conditions identical to those used in the determination of IFNγ. As positive controls for the release of IL-4 and IL-5 phytohemagglutinin was used (data not shown). These results are indicative of a selective induction of T helper cells with a Th1 phenotype. Fig. 2 shows also that the release of IL-2 was blocked when an anti-DR antibody was present in the medium. A tentative interpretation of these results could be that active peptides might be presented, probably by monocytes, to T helpers.

3.3. Expression of TNFα and IFNγ genes

We analyzed TNFα gene expression induced by immunopotentiating peptides searching its corresponding mRNA. After 3 h of incubation total RNA was isolated from lymphomononuclear cultures treated with peptide P8, Pa1, or, as negative controls, from cultures to which no peptides were added. The corresponding cDNA was obtained and used in the PCR experiments employing primers synthesized according to the published sequence for the TNFα gene [41]. The sequence of the sense was 5′-GTCAGATCATCTTCTCGAACC-3′, and that of the antisense 5′-CAGATAGATGGGCTCATACC-3′.

Amplified sequence corresponds to positions from 381 to 740 within the mRNA of TNFα. When cells were incubated for three h in the presence of either of the two active peptides indicated above, a clear band appeared on the gel corresponding to a cDNA of 360 bp showing that the expression of the TNFα gene had been induced. When RNA was extracted from cells incubated under the same conditions, but in the absence of active peptides, the equivalent band of cDNA was very weak, almost imperceptible.

INFγ gene expression was also investigated. After 6 h of incubation total RNA was isolated from lymphomononuclear cultures treated with peptides P8 or Pa1. For the PCR experiments primers were synthesized (sense, 5′-ATCGTTTTGGGTTCTCTTGG-3′. and antisense: 5′-TCTTTTGGATGCTCTGGTCA-3′) based on the published sequence of the INFγ gene [42], and amplifying a 235 bp fragment of the IFNγ cDNA. We found bands of this length on gels corresponding to cultures treated with actives peptides, whereas no bands of amplification was observed in control cultures. Since the fragment of the INFγ gene chosen for amplification contains in its genomic version an intron of 1300 bp the possibility that the amplified band could correspond to contaminating genomic DNA is easily discarded.

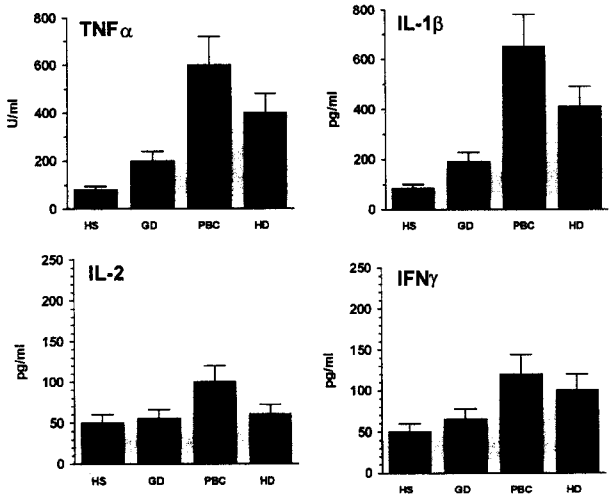

Fig. 3. Spontaneous release of cytokines by lymphomononuclear cells obtained from patients of autoimmune diseases. Values represent the mean of duplicates of independent experiments using blood from 7 patients of Graves' disease (GD), 4 patients of Hashimoto's disease (HD) and 4 of primary biliary cirrhosis (PBC). The differences between cells obtained from patients and healthy subjects (HS) were statistically significant ($P < 0.001$). At the moment of the blood extraction the patients selected for these experiments had a high percentage of activated monocytes ($CD14^+ > 20\%$).

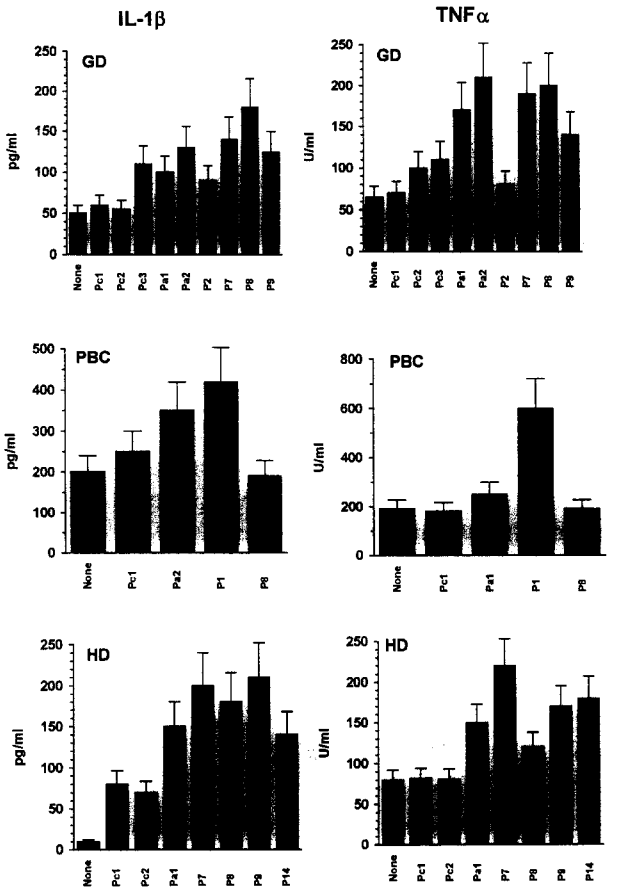

Fig. 4. Release of monocytic cytokines by lymphomononuclear cells from patients induced by specific immunopotentiating peptides. The patients selected for these determinations (5 of GD, 4 of HD and 4 of PBC) had at the moment of the blood extraction a percentage of cells expressing CD14 between 10 and 15. Experimental conditions were the same as in Figs. 4 and 1. The differences between cells treated with active peptides and control peptides or in the absence pf peptides were statistically significant ($P < 0.001$).

3.4. Cytokine release by lymphomononuclear cells belonging to patients of autoimmune diseases

We have also studied the spontaneous release of cytokines when lymphomononuclear cells obtained from patients of autoimmune diseases were incubated in the absence of immunomodulating peptides. Fig. 3 shows the spontaneous release of cytokines TNFα, IL-1β, IL-2 and IFNγ by lymphomononuclear cells from patients of GD, HD and PBC. All the patients included in this study had at the moment of the extraction a high percentage of acti-

vated monocytes ($CD14^+ > 20$), i.e. a value much higher than the average found in healthy subjects ($CD14^+ < 10$). Lymphomononuclear cells from all the patients studied released to the incubation medium the four cytokines tested in amounts considerably higher than those released by cells belonging to healthy individuals. It is also worth noting that the amount of the cytokines released by cells belonging to patients was similar to that released by cells obtained from healthy individuals, if incubations of the latter were carried out in the presence of immunopotentiating peptides containing a '2-6-11' motif. In no case IL-4 could be detected in the medium. These cells released this cytokine when incubated in the presence of phytohemagglutinin (data not shown).

Fig. 5 show the release of cytokines by the lymphomononuclear population belonging to patients of GD, HD or PBC, when incubations were carried out in the presence of one of the active peptides including that related to the autoantigen corresponding to each disease. All the patients selected for this experiment showed an expression of CD14 in approx. 15% of the cells of the entire lymphomononuclear population. It may be seen that active peptides stimulated the release of cytokines TNFα, IL-1β, (Fig. 4), IL-2 and IFNγ (Fig. 5). When a peptide related to the autoantigen characteristic of GD, PBC or HD matched the lymphomonuclear cells belonging to patients of the corresponding autoimmune disease, the amounts of cytokines released were higher than those obtained with other active peptides.

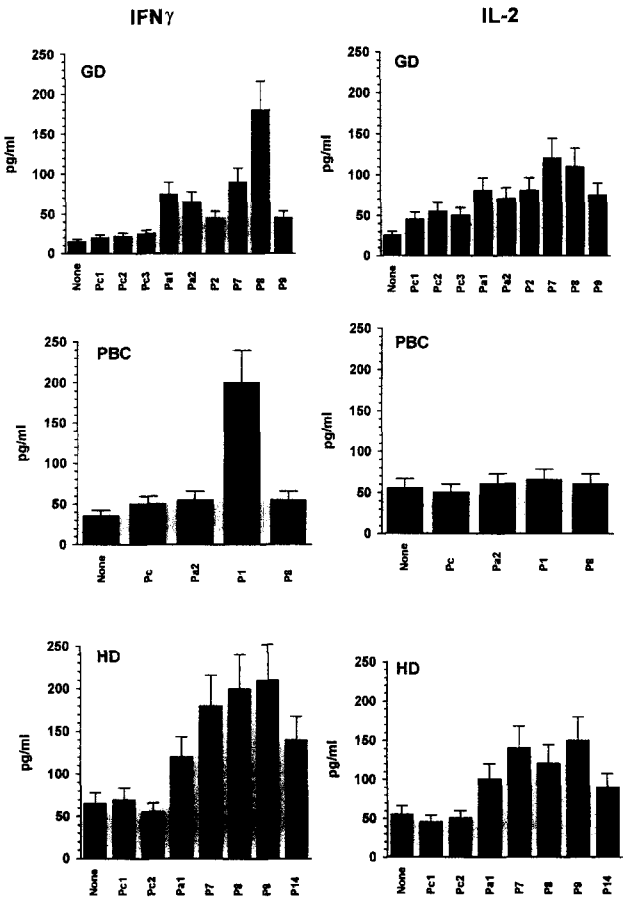

Fig. 5. Release of IFNγ and IL-2 by lymphomononuclear cells from patients induced by specific immunopotentiating peptides. The patients selected for these experiments were the same as those selected for the experiments summarized in Fig. 4. Experimental conditions were the same as in Fig. 2. The differences between cells treated with active peptides and control peptides or in the absence of peptides were statistically significant ($P < 0.001$).

In order to check that the higher spontaneous cytokine release by the cells obtained from patients was not due to the lysis of these cells, but to a higher cytokine production, we carried out the determination of cytokines released to the medium and then after the complete disruption of the cells. Lymphomononuclear cells freshly obtained from healthy subjects were resuspended in the incubation medium and disrupted in a nitrogen bomb. Neither TNFα nor IFNγ were detected. However, TNFα and IFNγ were detected when an equivalent number of cells obtained from a patient and treated under the same condition. Lymphomononuclear cells from healthy subjects were incubated for 24 h in the presence of active peptides Pa1 and P8. Both IFNγ and TNFα were released to the medium. Neither of these two cytokines were detected after removing the medium and disrupting the cells with a nitrogen bomb. Moreover, when cells from a patient with GD were incubated in the absence of immunopotentiating peptides for 24 h cytokines were released to the medium, and only negligible amounts of cytokines could be detected after removing the medium and replacing it with fresh medium and disrupting the cells. We have also studied the release of IL-4 and IL-5 to the medium and their possible retention by the cells after incubating them for 24 h in the presence of PHA. The amount of the cytokines retained by the cells was below 10% of the amount released.

4. Discussion

The striking presence of amino acid sequences enclosing a '2-6-11' motif in the autoantigens studied suggests that a presentation of fragments of them with sequences of this type could play a key role in the autoimmune response. A random search of unrelated human proteins permits us to assert that this structural pattern is not as universal as it might be thought, since many of them lack this motif altogether. Furthermore, for a protein to behave as an autoantigen or an immunopotentiating agent in autoimmunity, the presence of a specific motif or sequence would not be the sole prerequisite. The protein would also have to be processed in a way that at least one of the resulting peptides with the indicated motif could be presented by an APC, professional or nonprofessional.

We have also included in Table 1 three peptides with a serine residue at position 2: peptide P25, derived from KU (p70/p80) and peptides P48 and P49 from myelin-associ-

ated glycoprotein precursor. We propose this new variant of the '2-6-11' motif, since peptides of this type have also proved to be slightly active (Figs. 1 and 2).

As shown in Figs. 1 and 2 peptides having the active motif (fragments of autoantigens, viral and heat shock proteins) elicited a response consistent with a stimulation of monocytes and a presentation leading to a differentiation of T helpers towards a Th1 phenotype. Lymphomononuclear cells from patients of GD, HD or PBC spontaneously, i.e. without the addition of immunopotentiating peptides, released cytokines indicating an activation of monocytes and the presence of T helper cells with a Th1 phenotype. It has been described that monocytic cytokines, IL-1 and TNFα, are effector molecules in autoimmune disease [43]. It has also been shown that in the course of natural immunization some infectious agents expand human T cells with a stable phenotype, either Th1 or Th2, as defined by their typical profile of cytokine release. This seems to suggest that Th1 or Th2 phenotypes could represent properties of $CD4^+$ T cells selectively elicited by particular antigens [12,13]. Evidence now accumulating indicates that Th activity is involved in autoimmune diseases. Cytokines produced by Th1 cells, such as IFNγ and IL-2, have been implicated in the exacerbation of autoimmune processes. IFNγ might favor autoimmunity in tissues where the expression of HLA class II molecules is induced by this cytokine [21,22]; the possible involvement of IL-2 in autoimmune diseases has also been described [44]. A Th1 cytokine profile is prevalent in organ targets of patients with organ specific autoimmune diseases. The majority of $CD4^+$ cells isolated from thyroid infiltrates of patients of Hashimoto's thyroiditis or Graves'disease exhibited a Th1 phenotype and cytotoxic properties. A Th1 profile has also been found in $CD4^+$ T clones derived from retroorbital infiltrates in patients of Graves' ophtalmopathy and the cerebrospinal fluid of patients of multiple sclerosis (reviewed in [14]). However, a central role of Th2 type cells has been described in the pathogenesis of systemic autoimmunity; systemic autoimmune disease can be induced experimentally in rodents and this model share several features, such as MHC class II hyperexpression on B cells, hyper-IgE, increased IL-4 activity and impairment of IL-2 production [45].

We selected also a group of patients whose lymphomononuclear cells spontaneously released amounts of cytokines slightly higher than those belonging to healthy donors. When cells from patients were incubated in the presence of active peptides corresponding to their own autoantigens (Figs. 4 and 5) the ensuing release of cytokines reached a degree higher than that taking place with other active peptides, even those related to other autoantigens. The extent of the response generated by each peptide seems to depend on individual genetic factors, since cells obtained from patients (all of them with a high risk haplotype characteristic of the disease) exhibited a higher response to a specific peptide or peptides. This observation is not surprising, since, as now widely accepted, each MHC haplotype is capable of presenting a large variety of peptides, although with specific preferences [15–17]. A preference of this type could explain why susceptibility to an autoimmune disease is often linked to a particular HLA haplotype and/or specific T cell receptors (reviewed in [18–20]).

We suggest that a specific and precise triple interaction of HLA-II, peptides of this type and T cells could be involved in the pathogenesis of organ specific autoimmune diseases. Thus, circulating autoantigens or the protein of an infectious agent through molecular mimicry could be responsible for the stimulation of autoreactive cells, which, in turn, could cause the priming of autoreactive T-cells. It has been demonstrated that autoreactive B and T cells specific for autoantigens are components of the normal repertoire of lymphocytes [46]. There is also evidence that molecular mimicry could cause the disease [47]. A continuous stimulation of Th1 cells by these particular peptides having the '2-6-11' motif, and the associated release of IFNγ, would result in an activation of nonprofessional APCs, such as monocytes or tissue cells aberrantly expressing HLA-II; it is known that an aberrant expression of HLA-II takes place in tissues affected by the autoimmune disease a consequence of specific cytokines released by Th1 cells [21,22]. Once the stimulation of nonprofessional APCs has taken place new fragments of autoantigens, hidden until that moment, could now be presented (as reviewed in [23]). Autoreactive B and T cells, specific for 'self' 3 structures, would have then their way paved towards a potentiation of the autoimmune response and the establishment of a state of chronic autoimmunity.

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